EXAMPLE 3

Carbon-14 Labeled Cholesterol 5β, 6β-Epoxide

In a 25 ml. microflask fitted with a condenser 8 mg. (1 mCi, 20 μmol) of 4-^{14}C-cholesterol (50 mCi/mmol) and 75 mg ferric acetylacetonate in acetonitrile (10 ml) is treated dropwise with 30% hydrogen peroxide (0.5 ml) at 40°C with stirring. Excessive oxidant is destroyed with saturated aqueous sodium sulfite followed by extraction with ethyl ether (5 ml. x 3). Washing of the organic phase with saturated aqueous saline followed by drying with anhydrous sodium sulfate and vacuum evaporation of the solvent produces an amorphous residue. Silica gel gradient chromatography with benzene-acetone followed by recrystallization from aqueous acetone produces 4-^{14}C-cholesterol 5β, 6β-epoxide (4 mg., 50 mCi/mmol).

EXAMPLE 4

Tritium Labeled Cholesterol 5β, 6β-Epoxide

Following the procedure of Example 3, 5 mg (1 Ci, 13 μmol) of 1,2,6,7-^{3}H-cholesterol (75 Ci/mmol) and 50 mg ferric acetylacetonate in acetonitrile (10 ml) is treated dropwise with 30% hydrogen peroxide (0.3 ml) at 40°C with stirring. After chromatographic purification and recrystallization 1,2,6,7-^{3}H-cholesterol 5β, 6β-epoxide (3 mg., 75 Ci/mmol) is obtained. The radiolabeled cholesterol epoxides are diluted with unlabeled material to the desired specific activity.

EXAMPLE 5

3β, 5α-Dihydroxycholestan-6β-S-yl-Glutathione (Hapten)

To a solution of cholesterol 5α, 6α-epoxide (100 mg., 0.25 mmol) in ethanol (10 ml) is added glutathione (150 mg., 0.5 mmol) in water (5 ml). After addition of 5N sodium hydroxide (0.5 ml), the mixture is refluxed for 3 hours. After cooling, acidification with glacial acetic acid, and

vacuum evaporation, the residue is dissolved in 1% aqueous acetic acid (5 ml) and extracted with water saturated 1-butanol (10 ml x 3). Evaporation of the solvent produces a residue which is dissolved in water (5 ml) and is purified over an Amberlite XAD-2 column (40 x 2 cm) processed initially with successive 10 bed volumes of ethanol, methanol, water and methanol-water (1:1, v/v) washes. After addition of the reaction product the column is washed with water, methanol-water (1:1 v/v) and eluted with methanol (5:2:5 bed volumes, respectively). Evaporation of the solvent from fractions monitored by the ninhydrin reaction and thin layer chromatography on silica gel G60 plates with the solvent system, 1-butanol-gl. acetic acid-water (4:1:5, v/v/v) produces an amorphous residue (145 mg.) exhibiting a single ninhydrin-positive component.

EXAMPLE 5A

Biotransformation of Cholesterol 5α, 6α-Epoxide to 3β, 5α-Dihydroxycholestan-6β-S-yl-Glutathione

Cholesterol 5α, 6α-epoxide (20 µg, 0.05 µmol) in human prostatic fluid (1 ml) is incubated at 37° for 30 min. with a soluble rat liver S-glutathione transferase 8 (10 mg.) in the presence of glutathione (6 mg., 20 µmol) in 0.1 M potassium phosphate buffer, pH 7.0 to a final volume of 10 ml. The reaction product, 3β, 5α-dihydroxycholestan-6β-S-yl-glutathione, is measurable either as a hapten by specific antibody reaction or by direct extraction and purification.

EXAMPLE 6

3β, 6β-Dihydroxycholestan-5α-S-yl-Glutathione (Hapten)

Following the procedure of Example 5, cholesterol 5β, 6β-epoxide (100 mg., 0.25 mmol) in water (5 ml) and refluxed for 3 hours after the addition of 5N sodium hydroxide (0.5 ml). Extraction of the reaction mixture

followed by purification on Amberlite XAD-2 as outlined in Example 5 yields an amorphous product (130 mg.) exhibiting a single ninhydrin-positive component on silica gel G-60 thin layer chromatography with the solvent system, 1-butanol-gl. acetic acid-water (4:1:5, v/v/v).

EXAMPLE 7

3β, 5α-Dihydroxycholestan-5β-S-yl-Cystein (Hapten)

Following the procedure of Example 5, cholesterol 5α, 6α-epoxide (100 mg., 0.25 mmol) in ethanol (10 ml.) is added to L-cysteine (60 mg., 0.50 mmol) in water (5 ml) and refluxed for 3 hours after the addition of 5N sodium hydroxide (0.5 ml). Extraction of the reaction mixture followed by chromatographic purification on Amberlite XAD-2 yields an amorphous product (105 mg.) exhibiting a single ninhydrin-positive component on silica gel G-60 thin layer chromatography with the solvent system, 1-butanol-formic acid-water (4:1:2, v/v/v).

EXAMPLE 8

3β, 6β-Dihydroxycholestan-5α-S-yl-Cysteine (Hapten)

Following the procedure of Example 5, cholesterol 5β, 6β-epoxide (100 mg., 0.25 mmol) in ethanol (10 ml.) is added to L-cysteine (60 mg., 0.50 mmol) in water (5 ml) and refluxed for 3 hours after the addition of 5N sodium hydroxide (0.5 ml). Extraction of the reaction mixture followed by chromatographic purification on Amberlite XAD-2 yields an amorphous product (98 mg.) exhibiting a single ninhydrin-positive component on silica gel G-60 thin layer chromatography with the solvent system, 1-butanol-formic acid-water (4:1:2, v/v/v).

EXAMPLE 9

3β, 5α-Dihydroxycholestan-6β-S-yl-Thiophenol (Hapten)

In a 50 ml flask fitted with a condenser cholesterol 5α, 6α-epoxide (100 mg., 0.25 mmol) in benzene (10 ml) solution is treated dropwise with a benzene (10 ml) solution of thiophenol (55 mg., 0.5 mmol) containing a few drops of concentrated phosphoric acid. The mixture is refluxed for 1 hour. After cooling, the reaction mixture is evaporated under vacuum to an oily residue which is redissolved in ethyl ether (25 ml). The resultant solution is extracted with 5% aqueous sodium carbonate solution (10 ml. x 2), dried with anhydrous sodium sulfate, and evaporated under vacuum. The resultant residue is purified by liquid chromatography on silica gel G-60 employing chloroform-methanol gradient elution. Combination of fractions containing the desired product followed by vacuum evaporation produces an amorphous substance (85 mg) exhibiting a single component by ultraviolet absorption on silica gel G-60 thin layer chromatographic plates after development with the solvent system, 1-butanol-gl. acetic acid-water (3:1:5, v/v/v).

EXAMPLE 10

3β, 6β-Dihydroxycholestan-5α-S-yl-Thiophenol (Hapten)

Following the procedure of Example 9 cholesterol 5β, 6β-epoxide (100 mg., 0.25 mmol) in benzene (10 ml) solution is treated with a benzene (10 ml) solution of thiophenol (55 mg., 0.5 mmol) containing a few drops of concentrated phosphoric acid. The reaction mixture is found to contain 3β, 6β-dihydroxycholestan-5α-S-yl-thiophenol which is recovered by the procedure outlined in Example 9. The amorphous product (35 mg) exhibits a single ultraviolet-absorbing component on silica gel thin layer chromatography with the solvent system, 1-butanol-gl. acetic acid-water (3:1:5, v/v/v).

EXAMPLE 11

3β, 5α-Dihydroxycholestan-6β-S-yl-O-Thiocresol (Hapten)

Following the procedure of Example 9 cholesterol 5α, 6α-epoxide (100 mg., 0.25 mmol) is treated with O-thiocresol (60 mg., 0.50 mmol), and the desired product, 3β, 5α-dihydroxycholestan-6β-S-yl-O-thiocresol, is recovered as an amorphous solid (75 mg.)

EXAMPLE 12

3β, 6β-Dihydroxycholestan-5α-S-yl-O-Thiocresol (Hapten)

Following the procedure of Example 9 cholesterol 5β, 6β-epoxide (100 mg., 0.25 mmol) is treated with O-thiocresol (60 mg., 0.50 mmol), and the desired product, 3β, 6β-dihydroxycholestan-5α-S-yl-O-thiocresol, is recovered as an amorphous solid (40 mg.)

EXAMPLE 13

3β, 5α-Dihydroxycholestan-6β-S-yl-m-Thiocresol (Hapten)

Following the procedure of Example 9 cholesterol 5α, 6α-epoxide (100 mg., 0.25 mmol) is treated with m-thiocresol (60 mg., 0.50 mmol), and the desired product, 3β, 5α-dihydrocholestan-6β-S-yl-m-thiocresol, is recovered as an amorphous solid (72 mg.).

EXAMPLE 14

3β, 6β-Dihydroxycholestan-5α-S-yl-m-Thiocresol (Hapten)

Following the procedure of Example 9 cholesterol 5β, 6β-epoxide (100 mg., 0.25 mmol) is treated with m-thiocresol (60 mg., 0.50 mmol), and the desired product, 3β, 6β-dihydroxycholestan-5α-S-yl-m-thiocresol, is recovered as an amorphous solid (30 mg.)

EXAMPLE 15

3β ,6β-Dihydroxycholestan-6β-S-yl-p-thiocresol (Hapten)

Following the procedure of Example 9 cholesterol 5α, 6α-epoxide (100 mg., 0.25 mmol) is treated with p-thiocresol (60 mg., 0.50 mmol), and the desired product, 3β, 5α-dihydrocholestan-6β-S-yl-p-thiocresol, is recovered as an amorphous solid (80 mg.).

EXAMPLE 16

3β, 6β-Dihydroxycholestan-5α-S-yl-p-Thiocresol (Hapten)

Following the procedure of Example 9 cholesterol 5β, 6β-epoxide (100 mg., 0.25 mmol) is treated with p-thiocresol (60 mg., 0.50 mmol), and the desired product, 3β, 6β-dihydroxycholestan-5α-S-yl-p-thiocresol, is recovered as an amorphous solid (38 mg.)

EXAMPLE 17

3β, 5α-Dihydroxycholestan-6β-S-yl-Thioglycolic Acid (Hapten)

In a 50 ml flask fitted with a condenser cholesterol 5α, 6α-epoxide (100 mg., 0.25 mmol) in ethanol (10 ml) solution is refluxed for 2 hours with thioglycolic acid (46 mg., 0.50 mmol) dissolved in 0.5N aqueous sodium hydroxide (5 ml). After cooling, the reaction mixture is acidified with glacial acetic acid and evaporated under vacuum. The oily residue is extracted with benzene (5 ml x 3), and the combined extracts dried with anhydrous sodium sulfate. After vacuum evaporation, the residue is purified by silica gel G-60 liquid column chromatography employing chloroform-methanol gradient elution. The product, 3β, 5α-dihydroxycholestan-6β-S-yl-thioglycolic acid, is obtained as an amorphous solid (80 mg.) from evaporation of selective chromatographic fractions.

EXAMPLE 18

3β, 5β-Dihydroxycholestan-5α-S-yl-Thioglycolic Acid (Hapten)

Following the procedure of Example 17 cholesterol 5β, 6 -epoxide (100 mg., 0.25 mmol) is treated with thioglycolic acid (46 mg., 0.50 mmol) in 0.5N sodium hydroxide solution (5 ml). After extraction and silica gel liquid chromatography with chloroform-methanol gradient elution, the product, 3 , 6 -dihydroxycholestan-5α-S-yl-thioglycolic acid, is obtained from selected fractions upon evaporation as an amorphous solid (33 mg.).

EXAMPLE 19

3β, 5α-Dihydroxycholestan-6β-S-yl-Thiolactic Acid (Hapten)

Following the procedure of Example 17 cholesterol 5α, 6α-epoxide (100 mg., 0.25 mmol) is treated with thiolactic acid (53 mg., 0.50 mmol) in 0.5N sodium hydroxide solution (5 ml). Upon extraction and liquid chromatographic purification the product, 3β, 5α-dihydroxycholestan-6β-S-yl-thiolactic acid, is obtained from selected fractions upon evaporation as an amorphous solid (75 mg.)

EXAMPLE 20

3β, 6β-Dihydroxycholestan-5α-S-yl-Thiolactic Acid (Hapten)

Following the procedure of Example 17 cholesterol 5β, 6β-epoxide (100 mg., 0.25 mmol.) is treated with thiolactic acid (53 mg., 0.50 mmol) in 0.5N sodium hydroxide solution (5 ml). After extraction and chromatographic purification the product, 3β, 6β-dihydroxycholestan-5α-S-yl-thiolactic acid, is obtained from selected fractions as an amorphous solid (30 mg.).

EXAMPLE 21

3β, 5α-Dihydroxycholestan-6β-S-yl-Thiosalicyclic Acid (Hapten)

Following the procedure of Example 17 cholesterol 5α, 6α-epoxide (100 mg., 0.25 mmol) is treated with thiosalicyclic acid (77 mg., 0.50 mmol) in 0.5N sodium hydroxide solution (5 ml). After extraction, chromatographic purification, and evaporation of selected fractions, the product, 3β, 5α-dihydroxycholestan-6β-S-yl-thiosalicyclic acid, is obtained as a microcrystalline solid (110 mg.).

EXAMPLE 22

3β, 6β-Dihydroxycholestan-5α-S-yl-Thiosalicyclic Acid (Hapten)

Following the procedures of Example 17 cholesterol 5β, 6β-epoxide (100 mg., 0.25 mmol.) is treated with thiosalicyclic acid (77 mg., 0.50 mmol) in 0.5N sodium hydroxide solution (5 ml). After extraction, chromatographic purification, and evaporation of selected fractions, the product, 3β, 6β-dihydroxycholestan-5α-S-yl-thiosalicyclic acid, is obtained as a semicrystalline solid (43 mg.).

EXAMPLE 23

3β, 5α-Dihydroxycholestan-6β-S-yl-2-Thiouracil (Hapten)

Following the procedure of Example 17 cholesterol 5α, 6α-epoxide (100 mg., 0.25 mmol) is treated with 2-thiouracil (64 mg., 0.50 mmol) in 0.5N sodium hydroxide solution (5 ml). After extraction, chromatographic purification, and evaporation of selected fractions, the product. 3β, 5α-dihydroxycholestan-6β-S-yl-2-thiouracil, is obtained as a semicrystalline solid (101 mg.).

EXAMPLE 24

3β, 6β-Dihydroxycholestan-5α-S-yl-2-Thiouracil (Hapten)

Following the procedure of Example 17 cholesterol 5β 6β-epoxide (100 mg., 0.25 mmol) is treated with 2-thiouracil (64 mg., 0.50 mmol) in 0.5N sodium hydroxide solution (5N). After extraction, chromatographic purification, and evaporation of selected fractions, the product, 3β, 6β-dihydroxycholestan-5α-S-yl-2-thiouracil, is obtained as a semicrystalline solid (38 mg).

EXAMPLE 25

3β, 5α-Dihydroxycholestan-6β-O-p-Toluenesulfonate (Hapten)

In a 50 ml. flask fitted with a stirrer, cholesterol 5α, 6α-epoxide (100 mg., 0.25 mmol) in benzene (10 ml) solution is combined with p-toluenesulfonic acid (86 mg., 0.50 mmol) in benzene (10 ml) and stirred for 4 hours at room temperature. The reaction mixture is extracted with 5% aqueous sodium bicarbonate solution (5 ml x 3), followed by water washes and drying with anhydrous sodium sulfate. Vacuum evaporation of the solvent produces an oily residue. Purification with silica gel G-60 liquid chromatography employing chloroform-methanol gradient elution produces selected fractions containing the product, 3β, 5α-dihydroxycholestan-6β-O-p-toluenesulfonate. Upon vacuum evaporation the product is obtained as a semicrystalline solid (70 mg.).

EXAMPLE 26

3β, 6β-Dihydroxycholestan-5α-O-p-Toluenesulfonate (Hapten)

Following the procedure of Example 25 cholesterol 5β-6β-epoxide and p-toluenesulfonate are combined in 1:2 molar ratio. After reaction the product, 3β, 6β-dihydroxy-cholestan-5α-O-p-toluenesulfonate, is purified by silica gel chromatography and recovered as a semicrystalline solid.

EXAMPLE 27

3β, 5α-Dihydroxycholestan-6β-O-Trifluoroacetate (Hapten)

Following the procedure of Example 25 cholesterol 5α, 6α-epoxide and trifluoroacetic acid are combined in 1:2 molar ratio. After reaction the product, 3β, 5α-dihydro-cholestan-6β-O-trifluoroacetate, is purified by silica gel chromatography and recovered as an amorphous solid.

EXAMPLE 28

3β, 6β-Dihydroxycholestan-5α-O-Trifluoroacetate (Hapten)

Following the procedure of Example 25 cholesterol 5β, 6β-epoxide and trifluoroacetic acid are combined in 1:2 molar ratio. After reaction the product, 3β, 6β-dihy-droxycholestan-5α-O-trifluoroacetate, is purified by silica gel chromatography and recovered as an amorphous solid.

EXAMPLE 29

3β, 5α-Dihydroxycholestan-6β-N-yl-Imidazole (Hapten)

In a 50 ml. flask fitted with a stirrer cholesterol 5α, 6α-epoxide (100 mg., 0.25 mmol) in ethanol (10 ml) solution is combined with imidazole (35 mg., 0.5 mmol) in ethanol

(10 ml). The reaction mixture is stirred at 80°C for 4 hours. Upon vacuum evaporation of the solvent an oil residue remains. Silica gel G-60 liquid column chromatography with chloroform-methanol gradient elution provides fractions containing the imidazole adduct product of cholesterol 5α, 6α-epoxide. Upon evaporation of the solvents under vacuum an amorphous product (41 mg.) is produced.

EXAMPLE 30

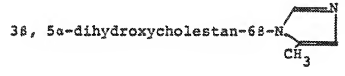
3β, 6β-Dihydroxycholestan-5α-N-yl-Imidazole (Hapten)

Following the procedure of Example 29 cholesterol 5β, 6β-epoxide and imidazole in 1:2 molar ratio interact to form the desired product which is recovered.

EXAMPLE 31

Cholesterol 5α, 6α-Epoxide-α-Methyl Imidazole Adduct (Hapten)

Following the procedure of Example 29 cholesterol 5α, 6α-epoxide and α-methylimidazole in 1:2 molar ratio produce the desired product.



EXAMPLE 32

Cholesterol 5β, 6β-Epoxide-α-Methyl Imidazole Adduct (Hapten)

Following the procedure of Example 29 cholesterol 5β, 6β-epoxide and α-methyl imidazole in 1:2 molar ratio produce the desired product.

3β, 6β-dihydroxycholestan-5α- 

EXAMPLE 33

Cholesterol 5α, 6α-Epoxide-α, β-Imidazole Dicarboxylic Acid Adduct (Hapten)

Following the procedure of Example 29 cholesterol 5α, 6α-epoxide and α, β-imidazole dicarboxylic acid in 1:2 molar ratio under alkaline conditions produce the desired product.

3β, 5α-dihydroxycholestan-6β- 

EXAMPLE 34

Cholesterol 5β, 6β-Epoxide-α, β-Imidazole Dicarboxylic Acid Adduct (Hapten)

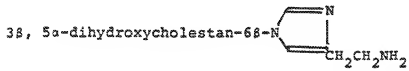
Following the procedure of Example 33 cholesterol 5β, 6β-epoxide and α,β-imidazole dicarboxylic acid in 1:2 molar ratio produce the desired product.

3β, 6β-dihydroxycholestan-5α- 

EXAMPLE 35

Cholesterol 5α, 6α-Epoxide-Histamine Adduct (Hapten)

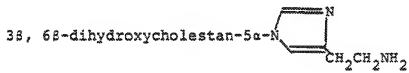
Following the procedure of Example 29 the desired product is obtained from cholesterol 5α, 6α-epoxide and histamine.



EXAMPLE 36

Cholesterol 5ß, 6ß-Epoxide-Histamine Adduct (Hapten)

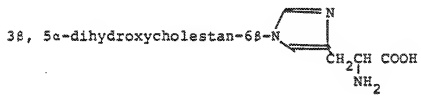
Following the procedure of Example 29 the desired product is obtained from cholesterol 5ß, 6ß-epoxide and histamine.



EXAMPLE 37

Cholesterol 5α, 6α-Epoxide-Histadine Adduct (Hapten)

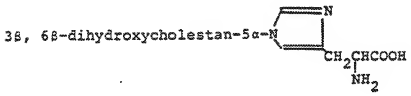
Following the procedure of Example 29 the desired product is obtained from cholesterol 5α, 6α-epoxide and L-histadine.



EXAMPLE 38

Cholesterol 5β, 6β-Epoxide-Histadine Adduct (Hapten)

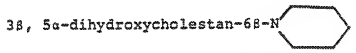
Following the procedure of Example 29 the desired product is obtained from cholesterol 5β, 6β-epoxide and L-histadine.



EXAMPLE 39

Cholesterol 5α, 6α-Epoxide-Piperidine Adduct (Hapten)

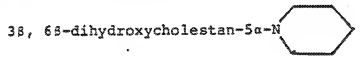
Following the procedure of Example 29 in either aqueous or aqueous-alcoholic solution the interaction of cholesterol 5α, 6α-epoxide and piperidine results in the desired product.



EXAMPLE 40

Cholesterol 5β, 6β-Epoxide-Piperidine Adduct (Hapten)

Procedure of Example 29 in aqueous or aqueous-alcoholic solution provides:



EXAMPLE 41

Cholesterol 5α, 6α-Epoxide-Alkyl Piperidine Adduct (Hapten)

Procedure of Example 29 in aqueous or aqueous-alcoholic solution provides:

3β, 5α-dihydroxycholestan-6β-N 

EXAMPLE 42

Cholesterol 5β, 6β-Epoxide-Alkyl Piperidine Adduct (Hapten)

Procedure of Example 29 in aqueous or aqueous-alcohol solution provides:

3β, 6β-dihydroxycholestan-5α-N 

EXAMPLE 43

Cholesterol 5α, 6α-Epoxide-Pipecolic Acid Adduct (Hapten)

Following the procedure of Example 29 in alkaline aqueous or aqueous-alcoholic solution.

3β, 5α-dihydroxycholestan-6β-N 

EXAMPLE 44

Cholesterol 5ß, 6ß-Epoxide-Pipecolic Acid Adduct (Hapten)

Following the procedure of Example 29 in alkaline aqueous or aqueous-alcoholic solution.

3ß, 6ß-dihydroxycholestan-5α-N 

EXAMPLE 45

Cholesterol 5α, 6α-Epoxide-Pyrrolidine Adduct (Hapten)

Following the procedure of Example 29 in aqueous or aqueous-alcoholic solution.

3ß, 5α-dihydroxycholestan-6ß-N 

EXAMPLE 46

Cholesterol 5ß, 6ß-Epoxide-Pyrrolidine Adduct (Hapten)

Following the procedure of Example 29 in aqueous or aqueous-alcoholic solution.

3ß, 6ß-dihydroxycholestan-5α-N 

EXAMPLE 47

Cholesterol 5α, 6α-Epoxide-3-Pyrroline Adduct (Hapten)

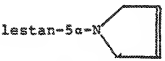
Following the procedure of Example 29 in aqueous or aqueous-alcoholic solution.

3ß, 5α-dihydroxycholestan-6ß-N 

EXAMPLE 48

Cholesterol 5β, 6β-Epoxide-3-Pyrroline Adduct (Hapten)

Following the procedure of Example 29 in aqueous or aqueous-alcoholic solution.

3β, 6β-dihydroxycholestan-5α-N 

EXAMPLE 49

Cholesterol 5α, 6α-Epoxide-Amino Acid Adducts (Hapten)

Following the procedure of Example 29 in aqueous or aqueous-alcoholic solution with neutral-alkaline conditions a variety of amino acids can serve as nucleophiles.

3β, 5α-dihydroxycholestan-6β-NH-CH-COOH
|
R

EXAMPLE 50

With Cholesterol 5β, 6β-Epoxide and Amino Acids

3β, 6β-dihydroxycholestan-5α-NH-CH-COOH
|
R

EXAMPLE 51

6β-N-Propxy-3β, 5α-Dihydroxycholestane (Hapten)

In a flask (50 ml) fitted with a condenser cholesterol 5α, 6α-epoxide (100 mg., 0.25 mmol) in 1-propanol (20 ml) solution containing trifluoroacetic acid (1.0 ml) is refluxed for 1 hour. With vacuum evaporation the solvent is removed. The oily residue is dissolved in benzene (10 ml), extracted with 5% aqueous sodium bicarbonate (2 ml x 2) and with water, and dried with anhydrous sodium sulfate. After vacuum evaporation the amorphous solid residue is purified by silica gel G-60 column liquid chromatography employing chloroform-methanol gradient elution. Selected

fractions provide the product, 6β-n-propoxy-3β, 5α-dihydroxycholestane. (45 mg).

EXAMPLE 52

5α-N-Butoxy-3β, 6β-Dihydroxycholestane (Hapten)

Following the procedure outlined in Example 51 cholesterol 5β, 6β-epoxide and 1-butanol with trifluoroacetic acid catalysis provides the product, 5α-m-butoxy-3β, 6β-dihydroxycholestane.

Other bulky alkyl alcohols can also be employed for interaction with the cholesterol epoxides to provide alkoxy haptens. Alkoxy groups bulkier than $-OCH_3$ would provide greater specificity with minimum to no cross-reactivity.

EXAMPLE 53

3β, 5α-Dihydroxycholestan-6β-N^6-Adenine (Hapten)

In a flask (50 ml) fitted with a stirrer cholesterol 5α, 6α-epoxide (100 mg., 0.25 mmol.) and adenine (135 mg., 1.0 mmol) dissolved in 50% aqueous ethanol (25 ml) are mixed at 37° for 24 hours. Upon evaporation under vacuum, the resultant reaction residue is extracted with benzene (10 ml. x 3). The combined benzene extract is washed with 1% aqueous ammonia and water, and dried with anhydrous sodium sulfate. After vacuum evaporation, the residue is purified by silica gel G-60 liquid chromatography with chloroform-methanol gradient elution. Selected fractions containing the N^6-adenine adduct are combined and evaporated under vacuum to yield an amorphous solid (11 mg) as the product.

EXAMPLE 54

3β, 6β-Dihydroxycholestan-5α-N^6-Adenine (Hapten)

Following the procedure of Example 53 cholesterol 5β, 6β-epoxide and adenine react to form the desired adduct product.

EXAMPLE 55

3β, 5α-Dihydroxycholestan-6β-N^2-Guanine (Hapten)

Following the procedure of Example 53 cholesterol 5α, 6α-epoxide and guanine react to form the desired adduct product involving the N^2 position of guanine.

EXAMPLE 56

3β, 6β-Dihydroxycholestan-5α-N^2-Guanine (Hapten)

Following the procedure of Example 53 cholesterol 5β, 6β-epoxide and guanine react to form the desired adduct product involving the N^2 position of guanine.

The interaction of various purines and pyrimidines and their respective nucleoside and nucleotide derivatives with cholesterol 5α, 6α-epoxide and cholesterol 5β, 6β-epoxide take place in aqueous or aqueous-alcohol solutions at neutrality producing, respectively, the 3β, 5α-dihydroxy-cholestan-6β- and the 3β, 6β-dihydroxycholestan-5α-adduct products. All of the positions of interaction on the purine and pyrimidine molecules are not fully known since mixtures most often result.

The different purines and position of interaction:

N^6 - adenine (some N^9 substitution)
N^6 - adenosine
N^6 - 3'-adenylic acid
N^6 - 5'-adenylic acid
N^6 - adenosine diphosphate
N^6 - adenosine triphosphate
N^6 - 2-methyladenine (some N^9 substitution)
N^2 - guanine (some N^9 substitution)
N^2 - guanosine (some N^7 substitution)
N^2-3' - guanylic acid (some N^7 substitution)
N^2-5' - guanylic acid (some N^7 substitution)
N^2-1 - methylguanine (some N^9 substitution)

Cholesterol Epoxide Bridge Compounds:

EXAMPLE 57

5α, 6α-Epoxycholestan-3β-O-Hemisuccinate

In a 500 ml. flask provided with a condenser cholesterol 5α, 6α-epoxide (10 gm., 25 mmol) is refluxed with succinic anhydride (5 gm., 50 mmol) in pyridine (100 ml) solution under nitrogen for 12 hours. After cooling benzene (300 ml) and crushed ice are added to the reaction mixture. The cooled solution is slightly acidified with cold aqueous hydrochloric acid with vigorous stirring. Thereafter the cold mixture is extracted with chloroform (100 ml x 3). The combined chloroform extracts are washed with water and dried with anhydrous sodium sulfate. Evaporation under vacuum of the chloroform produced an amorphous residue which was triturated with ethyl ether. The hemisuccinate product (6.2 gm) was dried after washing with ice-cold ether.

EXAMPLE 58

5β, 6β-Epoxycholestan-3β-O-Hemisuccinate

Following the procedure of Example 57 cholesterol 5β, 6β-epoxide (10 gm., 25 mmol) and succinic anhydride (5 gm., 50 mmol) interact to form the desired product (5.5 gm).

EXAMPLE 59

5α, 6α-Epoxycholestan-3β-O-Carboxymethyl Ether

In a 500 ml. flask provided with a condenser cholesterol 5α, 6α-epoxide (10 gm., 25 mmol) is refluxed with methyl bromoacetate (7 gm., 50 mmol) in pyridine (100 ml) solution under nitrogen for 8 hours. After cooling crushed ice is added to the reaction mixture and chloroform (300 ml) is then added. The chloroform layer is extracted with water washes and then evaporated under vacuum to produce an oily residue. Alcoholic potassium hydroxide (1%, 100 ml) is added to the reaction residue for saponification at 60° in a water bath for 1 hour. Addition of chloroform (100 ml) followed by aqueous washes and drying with anhydrous sodium sulfate produces upon vacuum evaporation an amorphous product (4.0 gm).

EXAMPLE 60

5β, 6β-Epoxycholestan-3β-O-Carboxymethyl Ether

Following the procedure of Example 59 cholesterol 5β, 6β-epoxide (10 gm., 25 mmol) and methyl bromoacetate (7 gm., 50 mmol) interact and form the desired product (4.8 gm.) after saponification.

EXAMPLE 61

5α-Hydroxycholestan-6β-S-yl-Thiophenol-3β-O-Hemisuccinate

Following the procedure of Example 9 5α, 6α-epoxycholestan-3β-O-hemisuccinate is treated with thiophenol in 1:2 molar ratio in benzene solution containing a trace of concentrated phosphoric acid as catalyst to yield the desired product.

EXAMPLE 62

6β-Hydroxycholestan-5α-S-yl-Thiophenol-3β-O-Carboxymethyl Ether

Following the procedure of Example 9 5β, 6β-epoxycholestan-3β-O-carboxymethyl ether is treated with thiophenol in 1:2 molar ratio in benzene solution containing a trace of concentrated phosphoric acid as catalyst to yield the desired product.

EXAMPLE 63

5α-Hydroxycholestan-6β-O-p-Toluenesulfonate-3β-O-Carboxymethyl Ether

Following the procedure of Example 25 5α, 6α-epoxycholestan-3β-O-carboxymethyl ether is treated with p-toluene sulfonic acid in 1:2 molar ratio in benzene solution to yield the desired product.

EXAMPLE 64

6β-Hydroxycholestan-5α-O-p-Toluenesulfonate-3β-O-Hemisuccinate

Following the procedure of Example 25 5β, 6β-epoxycholestan-3β-O-hemisuccinate is treated with p-toluenesulfonic acid in 1:2 molar ratio in benzene solution to yield the desired product.

EXAMPLE 65

5α-Hydroxycholestan-6β-N-yl-Imidazole-3β-O-Carboxymethyl Ether

Following the procedure of Example 29 5α, 6α-epoxycholestan-3β-O-carboxymethyl ether is treated with imidazole in 1:2 molar ratio in ethanol at alkaline reaction to yield the desired product.

EXAMPLE 66

6β-Hydroxycholestan-5α-O-Ethoxy-3β-O-Hemisuccinate

Following the procedure of Example 51 5β, 6β-epoxycholestan-3-β-O-hemisuccinate in ethanol solution is treated with trifluoroacetic acid to yield the desired product.

EXAMPLE 67

Bovine Serum Albumin-5α, 6α-Epoxycholestan-3β-O-Hemisuccinate Coupling (Immunogen)

A mixture of purified 5α, 6α-epoxycholestan-3β-O-hemisuccinate (100 mg) in dioxane (10 ml), 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide hydrochloride (100 mg) in water (5 ml) and crystalline bovine serum albumin (BSA, 200 mg) in 0.05N phosphate buffer, pH7.8 (10 ml) is stirred at room temperature for 24 hours. The reaction mixture is then dialyzed against water for 48 hours at 5° in the refrigerator. The non-permeable material retained after dialysis is then centrifuged at 12000 x g (20 min), and the supernatant is lyophilized, yielding a light product residue (160 mg). The product reveals no free hapten and contains on the average 9 residues of hapten to each BSA molecule. When necessary the steroid-protein complexes are also purified to remove free hapten by G-25 sephadex gel filtration.

EXAMPLE 68

Bovine Serum Albumin-5ß, 6ß-Epoxycholestan-3ß-O-Hemisuccinate Coupling (Immunogen)

Following the procedure of Example 67 5ß, 6ß-epoxycholestan-3ß-O-hemisuccinate is coupled to bovine serum albumin, and the resultant steroid-protein complex is isolated and purified.

EXAMPLE 69

Bovine Serium Albumin-5α-6α-Epoxycholestan-3ß-O-Carboxymethyl Ether Coupling (Immunogen)

Following the procedure of Example 67 5α, 6α-epoxycholestan-3ß-O-carboxymethyl ether is coupled to bovine serum albumin, and the resultant steroid-protein complex is isolated and purified.

EXAMPLE 70

Bovine Serum Albumin-5ß, 6ß-Epoxycholestan-3ß-O-Carboxymethyl Ether Coupling (Immunogen)

Following the procedure of Example 67 5ß, 6ß-epoxycholestan-3ß-O-carboxymethyl ether is coupled to bovine serum albumin, and the resultant steroid-protein complex is isolated and purified.

EXAMPLE 71

Bovine Serum Albumin-6ß-Hydroxycholestan-5α-O-Ethoxy-3ß-O-Hemisuccinate Coupling (Immunogen)

Following the procedure of Example 67 6ß-hydroxycholestan-5α-O-ethoxy-3ß-O-hemisuccinate is coupled to bovine serum albumin, and the resultant steroid-protein complex is isolated and purified.

EXAMPLE 72

Bovine Serum Albumin-5α-Hydroxycholestan-6β-S-yl-Thiophenol-3β-O-Hemisuccinate Coupling (Immunogen)

Following the procedure of Example 67 5α-hydroxycholestan-6β-S-yl-thiopenol-3β-O-hemisuccinate is coupled to bovine serum albumin, and the resultant steroid-protein complex is isolated and purified.

EXAMPLE 73

Bovine Serum Albumin-5α-Hydroxycholestan-6β-N-yl-Imidazola-3β-O-Carboxymethyl Ether Coupling (Immunogen)

Following the procedure of Example 67 5α-hydroxycholestan-6β-N-yl-imidazole-3β-O-carboxymethyl ether is coupled to bovine serum albumin, and the resultant steroid-protein complex is isolated and purified.

EXAMPLE 74

Bovine Serum Albumin-5α-Hydroxycholestan-6β-S-yl-Glutathione-3β-O-Carboxymethyl Ether Coupled Adduct (Immunogen)

A mixture of purified 5α, 6α-epoxycholestan-3β-O-carboxymethyl ether (100 mg) in dioxane (10 ml), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (100 mg) in water (5 ml) and crystalline bovine serum albumin (200 mg) in 0.05N phosphate buffer, pH 7.8 (10 ml) is stirred at room temperature for 24 hours. The reaction mixture is then dialyzed against water for 48 hours at 5° in the refrigerator. The non-permeable fraction is then centrifuged at 12000 x g for 20 minutes. The supernatant is then treated with glutathione (300 mg) for 72 hours at 5° in the refrigerator. In an alternating procedure the supernatant is treated with glutathione in the presence of rat liver S-glutathione transferase B. According to the procedure of

Example 5A, after reaction the product is purified by dialysis and G-25 sephadex gel filtration.

EXAMPLE 75

Bovine Serum Albumin-6ß-Hydroxycholestan-5α-S-yl-Glutathione-3ß-O-Hemisuccinate Couple Adduct (Immunogen)

Following the procedures of Example 74, bovine serum albumin is coupled to 5ß, 6ß-epoxycholestan-3ß-O-hemisuccinate and then interacted with gluthathione either chemically or enzymatically to produce the product adduct immunogen.

Immunological Procedures:

Immunization - Antigen (steroid-BSA conjugate, 5 to 15 mg per animal) is dissolved in 2 ml saline and emulsified with an equal volume of complete Freund's adjuvant (CFA). This emulsion is injected once into multiple intradermal and subcutaneous sites along both sides of the back of 4-month-old male rabbits. The rabbits are bled weekly from the marginal ear vein, starting two weeks after the injection. Goats (mature females, intact or ovariectomized) receive 4 subcutaneous injections of 3 mg antigen emulsified in CFA at weekly intervals, followed by booster injections at 6 to 7 week intervals. Blood samples are drawn from the jugular vein 5 weeks after the first injection and two weeks after each booster injection. Undiluted sera are stored at 4°C for up to 9 months.

Radioimmunoassay - Sera are diluted with 0.05M Tris-HCl buffer (pH 8.0) containing 0.1M NaCl and 0.1% NaN_3 to the extent required, so that 40-45% of a fixed amount of the homologous tritiated steroid (12-18 pg) bound to antibody, as indicated by a preliminary titration. To 0.4 ml lots of the diluted serum placed in 10 x 75 mm disposable test tubes, varying amounts (0.5×10^{-11} to 10^{-8} g) of unlabeled hapten or of heterologous steroids are added in 0.1 ml of the same buffer (10 µg/ml ethanolic solutions of the cold

steroids are diluted with buffer to the required concentration). This mixture is incubated for 30 minutes at 0°C before adding a fixed amount (12 -18 pg) of the homologous tritiated steroid in 0.1 ml Tris buffer, and then kept for another 3 h at 0°C. (This "pre-emptive" method of adding the cold steroid or unknown sample to the antiserum before the labeled steroid in our hands slightly enhances the sensitivity of the assay, compared to the "equilibrium" technique of adding the two steroid species simultaneously). The remaining free steroid is then removed by adding 0.1 ml of a suspension of dextran-coated charcoal in Tris buffer (0.5% w/v Norit A activated charcoal and 0.05% w/v Dextran T20), stirring for 10 minutes at 0°C and centrifugation at 2200 x g for 20 minutes at 4°C. A portion (0.5 ml) of the supernatant is withdrawn into a counting vial containing Insta-Gel (Packard Instrument Co.) for determination of the bound radioactive steroid by liquid scintillation counting.

Immunization Procedure

A great variety of immunization procedures may be employed for the production of antisera to steroids. Common practice is to inject only adjuvants emulsions subcutaneously or intramuscularly, footpad injection (either subcutaneous or intradermal) and the intranodal route, although the latter method is complicated by the technical difficulty of locating and injecting a number of separate lymph nodes at open operation.

A preferred method is the multiple intradermal injection procedure in which the immunogen emulsion is injected at 40 or more sites spread over a considerable part of the body surface. Antibody response is relatively rapid and booster injections have little further effect.

While almost all routes of administration such as subcutaneous, intramuscular, intravenous, into the lymph nodes or footpads are applied in connection with subsequent booster injections, only the multiple-site intradermal immunization appears to yield satisfactory results without booster injections.

A great variety of animal species may be used for immunization, including rabbits, sheep, goats, and guinea pigs.

The preferred embodiments described above are not intended to be limiting. Variations in the materials and processes described will be apparent to those skilled in the art. Thus, the present invention is to be limited only by the scope of the appended claims.

What is Claimed is:

1. A method for determining the presence or concentration of cholesterol epoxide in a sample of fluid comprising:

contacting said sample with a hapten in the presence of a hapten-linking reagent to form a ring-opened 3,5(6)-trans-diaxial dihydroxycholestane-6(5)-yl-hapten adduct;

contacting said adduct containing sample with an antibody to said adduct in the presence of a measured amount labelled adduct; measuring the amount of labelled adduct bound to said antibody.

2. A method for determining the presence or concentration of cholesterol epoxide in a sample of fluid comprising:

contacting said sample with a hapten in the presence of a hapten-linking reagent to form a ring-opened trans-3,5(6)-trans-diaxial-dihydroxycholestane-6(5)-yl-hapten adduct;

contacting said adduct-containing sample with a measured amount of a labelled antibody to said adduct;

separating unbound labelled antibody from bound labelled antibody;

measuring the amount of labelled antibody bound to said adduct.

3. The method according to Claim 2 wherein said antibody is labelled by a substance which is colorimetrically measurable.

4. The method according to Claim 1 wherein said labelled adduct is labelled by a substance which is spectrophotometrically measurable.

5. The method according to Claim 1 or 2 wherein said labelled antibody or labelled adduct is labelled by a radioactive isotope.

6. The method according to Claim 1 or 2 wherein said hapten is comprised essentially of glutathione and said hapten-linking reagent comprises S-glutathione transferase.

7. An immunogen comprising a 3,5(6)-trans-diaxial-dihydroxy-cholestane-6(5)-yl-hapten adduct.

8. An immunogen according to Claim 7 wherein said adduct is linked through covalently bonded bridges to a protein.

9. An immunogen according to Claim 7 or 8 wherein said hapten comprises glutathione.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US85/02274

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [2]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT CL4: G01N; 33/53, 33/92, 33/531, 33/533, 33/534, 33/566;
US CL : 435/7,11,15; 436/501,543,545,546,547,548,71

SEE Attachme

II. FIELDS SEARCHED

Minimum Documentation Searched [4]	
Classification System	Classification Symbols
US	435/7,11,15 935/110 436/501,543,545,546,547,548,71,800,804,817, 822,823

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

CAS ONLINE
BIOSIS
REGISTRY

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	N, Urology, Volume 20 No. 3, issued 1982, A. Sporer et al, see pages 244-250, (Abstract only)	1-9
Y	N, Journal of Immunology, Volume 116 No. 2, issued February 1976, S.O'Neil et al, see pages 363-366	7-9
Y	N, Steroids, Volume 19, issued March 1972, H.R. Linder et al, see pages 357-375	1-9

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [3]
27 January 1986	13 FEB 1986
International Searching Authority [1]	Signature of Authorized Officer [20]
ISA/US	Patricia DeSantis

Form PCT/ISA/210 (second sheet) (October 1981)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
Y	N, Journal of Steroid Biochemistry, Vol. 9, issued 1978, T. Nambara et al, see pages 785-790	1-9
Y	JP,B, 50-88219 (Nippon Taketsu Kano) 15 September 1975, see abstract.	7-9
Y	JP,B, 54-58490 (Orientalyeast KK) 11 May 1979, see abstract.	1-9
Y, P	WO,A, 84/04817 (Boehringer Mannheim GMBH) 6 December 1984, see abstract	1-9

ATTACHMENT

I. CLASSIFICATION OF SUBJECT MATTER

INT CL^4: C12Q 1/60, 1/48

US CL : 436/800,804,817,822,823